

REMARKS

Further and favorable reconsideration is respectfully requested in view of the foregoing amendments and following remarks.

Summary of Telephone Interview

Applicants kindly thank the Examiner for the helpful comments provided during the telephone interview of April 22, 2008. During the interview, Applicants discussed limiting claim 1 to the compounds set forth as Example 9 and Example 33 of Applicants' specification. Applicants discussed that the Declaration submitted November 15, 2007, as well as the Tables provided in Applicants' specification, provide evidence that these compounds provide unexpected and superior results over the cited art. The Examiner indicated that limiting claim 1 to these particular compounds would likely overcome the obviousness rejection.

During the interview, Applicants also discussed the composition claims and method of use claims which depend from the compound claims. Applicants asserted that these claims should be rejoined if they are dependent upon otherwise allowable compound claims. The Examiner agreed that the composition claims would be allowed if the compound claims are allowed. The Examiner also agreed to review the method of use claims for patentability. However, in order to expedite allowance of the present application, Applicants have cancelled, without prejudice, the method of use claims, and plan to pursue these claims in a continuation application.

The Examiner kindly indicated that Applicants should file the amendments after final rejection. Accordingly, presented herewith are the amendments proposed during the telephone interview. Applicants again thank the Examiner for her kind consideration and helpful comments.

Claim Amendments

Claim 1 has been amended to limit the compounds to those set forth in Example 9 and Example 33 of Applicants' specification. Claims 2-4, 6, 8-15 and 19-24 have been cancelled, without prejudice.

No new matter has been added to the application by the above-discussed amendments.

Claims 1 and 5 remain pending.

Consideration After Final Rejection

Although this amendment is presented after final rejection, the Examiner is respectfully requested to enter the amendments and consider the remarks, as they place the application in condition for allowance. The Examiner is kindly reminded that she agreed to consider the amendments and remarks after final rejection, during the telephone interview of April 22, 2008.

Rejection Under 35 U.S.C. § 112, First Paragraph

The rejection of claims 1-6, 8-15 and 19-24 under 35 U.S.C. § 112, first paragraph, has been rendered moot in view of the claim amendments.

Patentability Arguments

The patentability of the present invention over the disclosures of the references relied upon by the Examiner in rejecting the claims will be apparent upon consideration of the following remarks.

Rejection Under 35 U.S.C. § 103(a)

The rejection of claims 1-6, 8-15 and 19-24 under 35 U.S.C. § 103(a) as being unpatentable over EP '840 and US '610 is respectfully traversed.

Applicants filed a Rule 1.132 Declaration on November 15, 2007. The Examiner indicates that Applicants' Declaration "does have some data for one compound. Just the phenyl." Although not acquiescing to the Examiner's position, in order to expedite allowance of the present application, Applicants have amended the claims to recite the compounds set forth in Examples 9 and 33 of the specification. In particular, these compounds contain phenyl as the "A" substituent.

As discussed in the responses of October 31, 2007 (page 12) and November 15, 2007, the PDE IV inhibitory efficacy ($IC_{50} = 0.084\mu M$) of the inventive compound is about 3 times higher than the PDE IV inhibitory efficacy ($IC_{50} = 0.25\mu M$) of the US '610 compound. The inventive compound discussed herein is the compound of Example 9 of Applicants' specification, now

clearly recited in amended claim 1. [The compound of Example 33 differs from the compound of Example 9 only in the substitution of the phenyl group with an F group.]

As stated in the prior response, it is clear that the presence or absence of methylene greatly affects PDE IV inhibition activity. Thus, the compounds recited in Applicants' amended claims provide unexpectedly superior PDE IV inhibition activity, which would not have been expected by one of ordinary skill in the art.

Applicants also refer to Appendix 1 (attached hereto), which shows that GPD-1116 (the compound of Example 9) markedly attenuates the development of emphysema (i.e. pulmonary emphysema). [Please note that Appendix 1 was published more than five years after the priority date of Applicants' application.]

The Examiner admits in the Office Action of May 31, 2007 that the difference in the structure of the cited art and the claimed compounds is the presence of the (CH₂)_m linker in Applicants' claimed compounds. As the Examiner is aware, MPEP 716.02(a)(II) states that "[e]vidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness. 'Evidence that a compound is unexpectedly superior in one of a spectrum of common properties . . . can be enough to rebut a *prima facie* case of obviousness.'"

As discussed above, the compounds recited in Applicants' amended claim 1 are unexpectedly superior in PDE IV inhibition activity when compared to the teachings of the cited art. Accordingly, assuming for the sake of argument, that the Examiner has presented a *prima facie* case of obviousness, Applicants have clearly rebutted any such showing according to MPEP 716.02(a)(II).

Applicants also note that the Examiner refers to two new references, WO '244 and EP '725, as providing additional motivation to modify the US '610 compounds. However, neither of these references remedy the deficiencies of EP '840 and US '610, as discussed above. In particular, neither of these references counter Applicants' showing of unexpected and superior results.

For these reasons, the invention of claims 1 and 5 is clearly patentable over the cited references.

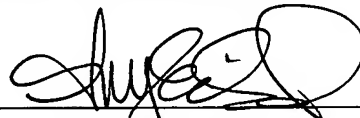
Conclusion

Therefore, in view of the foregoing amendments and remarks, it is submitted that each of the grounds of rejection set forth by the Examiner has been overcome, and that the application is in condition for allowance. Such allowance is solicited.

If, after reviewing this Amendment, the Examiner feels there are any issues remaining which must be resolved before the application can be passed to issue, the Examiner is respectfully requested to contact the undersigned by telephone in order to resolve such issues.

Respectfully submitted,

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Attachments: Appendix 1

Phosphodiesterase 4 inhibitor GPD-1116 markedly attenuates the development of cigarette smoke-induced emphysema in senescence-accelerated mice P1 strain

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Mori H, Nose T, Ishitani K, Kasagi S, Souma S, Akiyoshi T, Kodama Y, Mori T, Kondo M, Sasaki S, Iwase A, Takahashi K, Fukuchi Y, Seyama K. Phosphodiesterase 4 inhibitor GPD-1116 markedly attenuates the development of cigarette smoke-induced emphysema in senescence-accelerated mice P1 strain. *Am J Physiol Lung Cell Mol Physiol* 294: L196–L204, 2008. First published November 9, 2007; doi:10.1152/ajplung.00173.2007.—Phosphodiesterase 4 (PDE4) is an intracellular enzyme specifically degrading cAMP, a second messenger exerting inhibitory effects on many inflammatory cells. To investigate whether GPD-1116 (a PDE4 inhibitor) prevents murine lungs from developing cigarette smoke-induced emphysema, the senescence-accelerated mouse (SAM) P1 strain was exposed to either fresh air or cigarette smoke for 8 wk with or without oral administration of GPD-1116. We confirmed the development of smoke-induced emphysema in SAMP1 [air vs. smoke (means \pm SE); the mean linear intercepts (MLI), 52.9 ± 0.8 vs. 68.4 ± 4.2 μ m, $P < 0.05$, and destructive index (DI), $4.5\% \pm 1.3\%$ vs. $16.0\% \pm 0.4\%$, $P < 0.01$]. Emphysema was markedly attenuated by GPD-1116 (MLI = 57.0 ± 1.4 μ m, $P < 0.05$; DI = $8.2\% \pm 0.6\%$, $P < 0.01$) compared with smoke-exposed SAMP1 without GPD-1116. Smoke-induced apoptosis of lung cells were also reduced by administration of GPD-1116. Matrix metalloproteinase (MMP)-12 activity in bronchoalveolar lavage fluid (BALF) was increased by smoke exposure (air vs. smoke, 4.1 ± 1.1 vs. 40.5 ± 16.2 area/ μ g protein; $P < 0.05$), but GPD-1116 significantly decreased MMP-12 activity in smoke-exposed mice (5.3 ± 2.1 area/ μ g protein). However, VEGF content in lung tissues and BALF decreased after smoke exposure, and the decrease was not markedly restored by oral administration of GPD-1116. Our study suggests that GPD-1116 attenuates smoke-induced emphysema by inhibiting the increase of smoke-induced MMP-12 activity and protecting lung cells from apoptosis, but is not likely to alleviate cigarette smoke-induced decrease of VEGF in SAMP1 lungs.

protease; aging; apoptosis; oxidative stress; vascular endothelial growth factor

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is a disease state characterized by airflow limitation and is a global health problem in terms of morbidity, mortality, and economic burden (16). Chronic cigarette smoke exposure is the most important risk factor and induces chronic inflammation of the airways and lung parenchyma by recruiting and activating inflamma-

tory cells to release proteinases, particularly elastase from neutrophils and various metalloproteinases from alveolar macrophages. Accordingly, drugs that control the underlying inflammatory and destructive processes are required for the treatment of COPD.

Phosphodiesterases (PDEs) are intracellular enzymes that degrade cyclic nucleotides. Among many isozymes, phosphodiesterase 4 (PDE4) specifically degrades cAMP, a second messenger exerting inhibitory effects on many inflammatory cells. PDE4 is ubiquitously expressed among inflammatory and immune cells including neutrophils, CD8⁺ T cells, macrophages, mast cells, eosinophils, and airway epithelial cells (1, 17, 30). PDE4 inhibitors show suppressive effects on various in vitro responses, including cytokine production, cell proliferation and chemotaxis, release of inflammatory mediators, and NADPH oxidase activity (13). In this context, PDE4 inhibitors are expected to ameliorate various aspects of inflammatory processes and to be a potent therapeutic agent for cigarette smoke-induced emphysema.

Senescence-accelerated mouse (SAM) strains are naturally occurring animal models for accelerated aging after normal development and maturation (18, 19). SAMP1 substrain has been reported to be a model for senile lung since age-related air space enlargement without parenchymal destruction was demonstrated (12, 22). We (9) have recently shown that SAMP1 is a useful animal model for cigarette smoke-induced emphysema since a relatively short term of smoke exposure is sufficient to generate emphysema due to intrinsic factor of accelerating aging. In addition, we (9) have demonstrated that SAMP1 emphysema model can be applicable for an interventional experiment to prevent alveolar destruction since concomitant ingestion of tomato juice containing a potent antioxidant lycopene successfully prevented SAMP1 lungs from developing smoke-induced emphysema.

In this study, we investigated whether GPD-1116, a newly synthesized PDE4 inhibitor, prevents SAMP1 mice lungs from developing cigarette smoke-induced emphysema. With recent clarification and understanding of molecular and cellular mechanisms of alveolar destruction, it is now recognized that not only inflammation with proteolysis, but also interaction be-

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tween lung cell apoptosis and VEGF are important to maintain the integrity of parenchymal structure (2, 27). In this context, we investigated macrophage matrix metalloproteinase (MMP)-12 activity in bronchoalveolar lavage (BAL) fluid (BALF), apoptosis, and VEGF content after chronic cigarette smoke exposure for 8 wk to determine the underlying mechanisms by which GPD-1116 alleviates smoke-induced emphysema.

MATERIALS AND METHODS

Animals. SAMP1/Ka strain mice, 12–14 wk old, were provided from the Council for SAM Research, bred in Animal Research Facility of Juntendo University School of Medicine, and used throughout this study. All procedures were approved by the Animals Ethics Committee of Juntendo University or by the Animal Research Committee of Aska Pharmaceutical. All mice were maintained and used as we (9) have described previously.

Acute inhalation of LPS to mice. Inhalation of LPS (*Escherichia coli*, serotype 055:B5, Sigma, St. Louis, MO) was performed using a commercially available ultrasonic nebulizer (Atomsonicnebulizer 305; Atom Medical, Tokyo, Japan). Briefly, SAMP1 mice, 14 wk old, were exposed to LPS aerosols (300 µg/ml in saline) for 10 min in a container (width 29 mm × diameter 29 mm × height 19 mm) connected to an ultrasonic nebulizer at 6 l/min airflow and 3 ml/min exposure dose. Mice in the negative control group were exposed to saline under the same condition.

Chronic inhalation of cigarette smoke. Inhalation of cigarette smoke was performed using a commercially marketed, unfiltered cigarette (29 mg of tar and 2.5 mg of nicotine per cigarette, Peace; Japan Tobacco, Tokyo, Japan) and the Tobacco Smoke Inhalation Experiment System for Small Animals (model SIS-CS; Shibata Scientific Technology, Tokyo, Japan) as described previously (9). Briefly, SAMP1 mice (12 wk old) were exposed to 2.0% cigarette smoke (mass concentration of total particulate matter, 554 mg/m³) that was prepared by diluting the originally generated smoke with compressed air for 30 min/day for 5 days/wk for 8 wk. As a control for the inhalation of cigarette smoke, mice were subjected to the same experimental procedure under the same conditions described above, but air was delivered instead of cigarette smoke.

PDE4 inhibitor (GPD-1116) and its administration to mice. GPD-1116 is a novel PDE4 inhibitor (3-benzyl-5-phenyl-1*H*-pyrazolo[4,3-*c*][1,8]naphthyridin-4(5*H*)-one), which is created by Aska Pharmaceutical, Tokyo, Japan (Fig. 1). GPD-1116 was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na; Wako Pure Chemical Industries) solution and then orally administered to mice at a dose of 1 or 2 mg/kg (100 or 200 µg/ml solution was administered at 10

ml/kg) 30 min before LPS or cigarette smoke exposure, respectively. Vehicle (0.5% CMC-Na) was also administered to SAMP1 mice in the control groups.

BAL and morphometric evaluation of the lungs. At 3 h after LPS exposure or 48 h after the last cigarette smoke exposure, mice were weighed and anesthetized with intraperitoneal injection of pentobarbital sodium in saline (100 mg/kg body wt) and then killed by exsanguination through the left atrium with perfusion of PBS through the main pulmonary artery. BAL was performed three times with 0.5 ml of PBS, and then lungs were removed. The number of leukocytes and cell population and histopathological changes of the lungs were evaluated as described previously (9). Furthermore, the concentrations of VEGF and TNF-α and the activity of MMP-12 were measured as described below.

The change in air space size was assessed by the determination of the mean linear intercepts (MLI) according to the method described by Thurlbeck (23). Ten randomly selected fields in each section at ×100 magnification were used for the calculation of the MLI. The destructive index (DI) was calculated to evaluate the destruction of the alveolar wall (7). Ten randomly selected fields in each section at ×50 magnification were used to calculate the DI. A DI value of more than 10% was considered to have significant destruction of the lung parenchyma (22).

Evaluation of apoptosis by immunohistochemistry for single-strand DNA. Apoptosis of lung cells were determined with immunohistochemistry using a rabbit polyclonal antibody against the single-stranded DNA (ssDNA; Dako Cytomation) as described previously (9). The percentage of apoptotic cells (ratio of positively immunostained nuclei to total count of the nuclei present in the field at ×200 magnification) was determined in three different areas from three planes of the lung per mouse (9).

Preparation of cigarette smoke extract and cell culture. Cigarette smoke extract (CSE) was prepared by bubbling 20% diluted cigarette smoke into DMEM (Sigma) for 5 min: diluted cigarette smoke was generated and delivered into the DMEM solution by a tobacco smoke generator (model SG-200). CSE was then sterilized by filtration (0.45-µm pore size) and used immediately for the cell culture experiment.

RAW264.7 cells, murine monocyte/macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FCS, 100,000 U/ml penicillin, and 100 µg/ml streptomycin (designated as complete medium). RAW264.7 cells were cultured at 2×10^5 cells per well on a 12-well plate and then incubated for 72 h with complete medium, complete medium supplemented with 20% CSE (1 volume of the original CSE was diluted with 4 volumes of complete medium), or complete medium supplemented with 20% CSE and 1 µM GPD-1116. The cell culture supernatant was used for the determination of MMP-12 activity.

To examine the effect of GPD-1116 on the intracellular content of cAMP, RAW264.7 cells were cultured as described above for 72 h with or without 20% CSE. GPD-1116 at 1 µM was added either at the initiation of culture or 5 min before termination of cell culture. After the removal of culture medium, RAW264.7 cells were incubated with 0.1 M HCl at room temperature for 20 min and then scraped off from the plate with a cell scraper. cAMP content was determined using a commercially available kit (Cyclic AMP EIA Kit, Cayman Chemical) according to the manufacturer's instructions.

Casein zymography to determine MMP-12 activity. MMP-12 activity in BALF and culture supernatant of RAW264.7 cells was determined with casein zymography. The supernatant of BALF and RAW264.7 cell culture medium was obtained by ultracentrifugation at 11,100 g for 10 min at 4°C and then concentrated by using the Centricon-10 filtration units (Millipore, Bedford, MA) at 5,000 g for 60 min at 4°C. The samples (10 µl) were treated with equal volume of 2× sample buffer (Invitrogen, Carlsbad, CA), and then 15 µl of the mixture was applied onto 12% polyacrylamide gel containing 2.5%

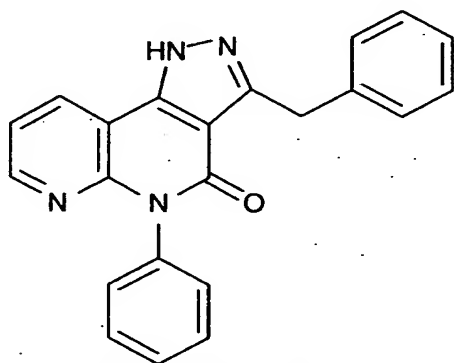


Fig. 1. Chemical structure of phosphodiesterase 4 (PDE4) inhibitor GPD-1116 is shown.

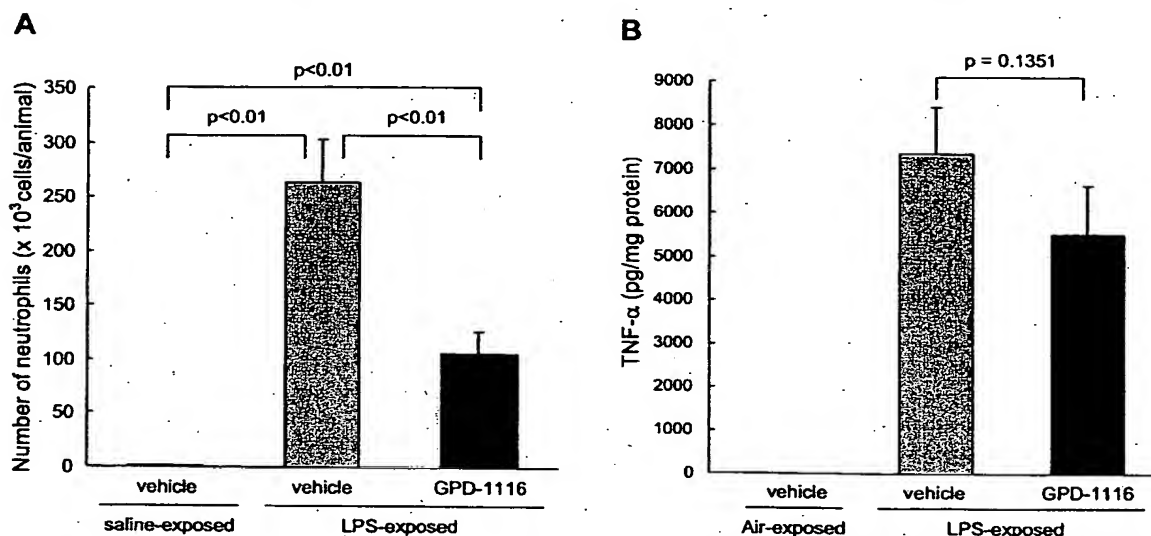


Fig. 2. A and B: changes in the number of neutrophils and the concentration of TNF- α in bronchoalveolar lavage fluid (BALF) of senescence-accelerated mouse (SAM) P1 strain (SAMP1) exposed to LPS aerosol. A: number of neutrophils in BALF ($n = 5$ in each group). Values are presented as means \pm SE (cells/animal). B: concentration of TNF- α in BALF ($n = 5$ in each group). Values are presented as means \pm SE (pg/mg protein). Statistical analysis between mice exposed to air and mice exposed to LPS with or without GPD-1116 was not performed since all the individual values in mice exposed to air were 0.

casein (Sigma) and electrophoresed at 4°C for 2 h. After electrophoresis, gels were washed twice for 30 min in 2% Triton X-100 at room temperature and incubated over two nights at 37°C in substrate buffer [50 mM Tris-HCl (pH 8.0), 5 mM CaCl₂, 0.5 μ M ZnCl₂]. Gels were stained with 1.25% Coomassie blue R-250 (Sigma) dissolved in 50% methanol and 10% acetic acid and destained with 10% methanol and 5% acetic acid. Caseinolytic activity was analyzed with a printgraph system (AE-6911; Atto, Tokyo, Japan) and a densitometer program (CS Analyzer, Atto).

Determination of TNF- α and VEGF concentrations in BALF and/or lung tissues. TNF- α concentration in BALF was determined using a commercially available ELISA kit (Quantikine Mouse TNF- α ELISA Kit, R&D Systems) according to the manufacturer's instructions.

The lung tissue homogenate was prepared to determine VEGF concentration as described previously (9). VEGF concentration in the BALF and supernatant of lung homogenate was determined using a commercially available ELISA kit (Quantikine Mouse VEGF Kit, R&D Systems) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using the unpaired *t*-test or Aspin-Welch *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of GPD-1116 on leukocyte influx into the BALF and TNF- α concentration in BALF when SAMP1 mice were exposed to LPS aerosol. As shown in Fig. 2, the number of neutrophils and TNF- α concentration in the BALF of SAMP1 mice exposed to LPS markedly and significantly increased (neutrophil, $263.8 \pm 39.9 \times 10^3$ cells/animal and TNF- α , $7,371 \pm 1,075$ pg/mg protein) compared with those of SAMP1 mice exposed to saline (neutrophil, $1.0 \pm 0.8 \times 10^3$ cells/animal, and TNF- α , 0.0 ± 0.0 pg/mg protein). GPD-1116, 1 mg/kg, exerted a significant inhibitory effect on the increase in number of neutrophils ($105.6 \pm 20.6 \times 10^3$ cells/animal; $P < 0.01$), and the inhibition rate was 60%. In addition, GPD-1116 tended to show an inhibitory effect on TNF- α production

($5,520 \pm 1,133$ pg/mg protein), but its effect was not statistically significant.

Effect of chronic cigarette smoke exposure on body weight and BALF in SAMP1 mice. SAMP1 mice were exposed to either fresh air with concomitant oral administration of vehicle (Group A), cigarette smoke with concomitant oral administration of vehicle (Group B), or cigarette smoke with concomitant oral administration of GPD-1116 (Group C); respectively, for 8 wk ($n = 6$ in each group). Body weights of SAMP1 mice did not change significantly among the three groups at initiation [Group A, 32.1 ± 0.8 g (mean \pm SE); Group B, 32.2 ± 0.6 g; and Group C, 32.1 ± 0.4 g, respectively] and after cigarette smoke exposure for 8 wk (Group A, 32.4 ± 0.3 g; Group B, 33.7 ± 1.6 g; and Group C, 33.7 ± 0.8 g, respectively). In addition, total cell number and cell populations in BALF did not differ significantly among the three SAMP1 groups (Table 1). Cell populations in BALF were composed mainly

Table 1. Cell populations in BALF after cigarette smoke exposure for 8 wk

Cell Populations	Total Cell Number, $\times 10^4$ cells/ml	Macrophages, %	Neutrophils, %	Lymphocytes, %
Group A	11.0 ± 0.1	98.5 ± 0.2	0	1.5 ± 0.2
Group B	15.8 ± 0.3	94.4 ± 2.5	0	5.6 ± 2.5
Group C	13.4 ± 0.1	97.4 ± 1.5	0	2.6 ± 1.5

Data are presented as means \pm SE ($n = 6$ for each group). Group A indicates senescence-accelerated mouse P1 strain (SAMP1) mice exposed to fresh air with concurrent oral administration of vehicle. Group B indicates SAMP1 mice exposed to cigarette smoke with concurrent oral administration of vehicle. Group C indicates SAMP1 mice exposed to cigarette smoke with concurrent oral administration of phosphodiesterase 4 inhibitor GPD-1116. There was no statistically significant difference in total cell number and cell populations between each group: Group A vs. B, $P = 0.0754$; Group A vs. C, $P = 0.1090$; and Group B vs. C, $P = 0.4513$. BALF, bronchoalveolar lavage fluid.

of macrophages in all groups, and both neutrophils and lymphocytes were barely detected even after cigarette smoke exposure for 8 wk.

Morphometric findings of the lungs in SAMP1 mice after cigarette smoke exposure. We evaluated the effect of GPD-1116 on the development of cigarette smoke-induced emphysema in SAMP1 mice (Fig. 3). After 8-wk exposure to cigarette smoke, the MLI significantly increased in the lungs of Group B SAMP1 mice (29%) compared with Group A SAMP1 mice (mean \pm SE, Group A vs. B, 52.9 ± 0.8 vs. 68.4 ± 4.2 μ m; $P < 0.05$; Fig. 3A). The DI also significantly increased in the lungs of Group B SAMP1 mice (more than 10%) compared with that of Group A (means \pm SE, Group A vs. B, $4.5\% \pm 1.3\%$ vs. $16.0\% \pm 0.4\%$; $P < 0.01$; Fig. 3B), indicating the development of smoke-induced emphysema in the lungs of Group B SAMP1 mice. However, GPD-1116 markedly inhibited air space enlargement (by 74%) induced by cigarette

smoke exposure for 8 wk (Group C, MLI = 57.0 ± 1.4 μ m; $P < 0.05$; Fig. 3A) as well as significantly attenuated the cigarette smoke-induced destruction of the lung parenchyma (by 68%) (Group C, DI = $8.2\% \pm 0.6\%$; $P < 0.01$; Fig. 3B). Representative histopathological microphotographs of the lungs from the three groups are presented in Fig. 3C.

Determination of apoptosis of lung cells in SAMP1 mice after cigarette smoke exposure. We investigated the effect of GPD-1116 on apoptosis of lung cells. Immunohistochemical examination with anti-ssDNA antibody revealed that apoptosis was widely detected in bronchial and bronchiolar epithelial cells and alveolar septal cells after cigarette smoke exposure for 8 wk (Group A vs. B; $P < 0.01$) but decreased by concurrent administration of GPD-1116 (Group B vs. C; $P < 0.01$; Fig. 4). When apoptotic nuclei of lung cells were counted in three areas, bronchial cells in the central airways, bronchiolar cells adjacent to the alveolar duct, and alveolar septal cells in the

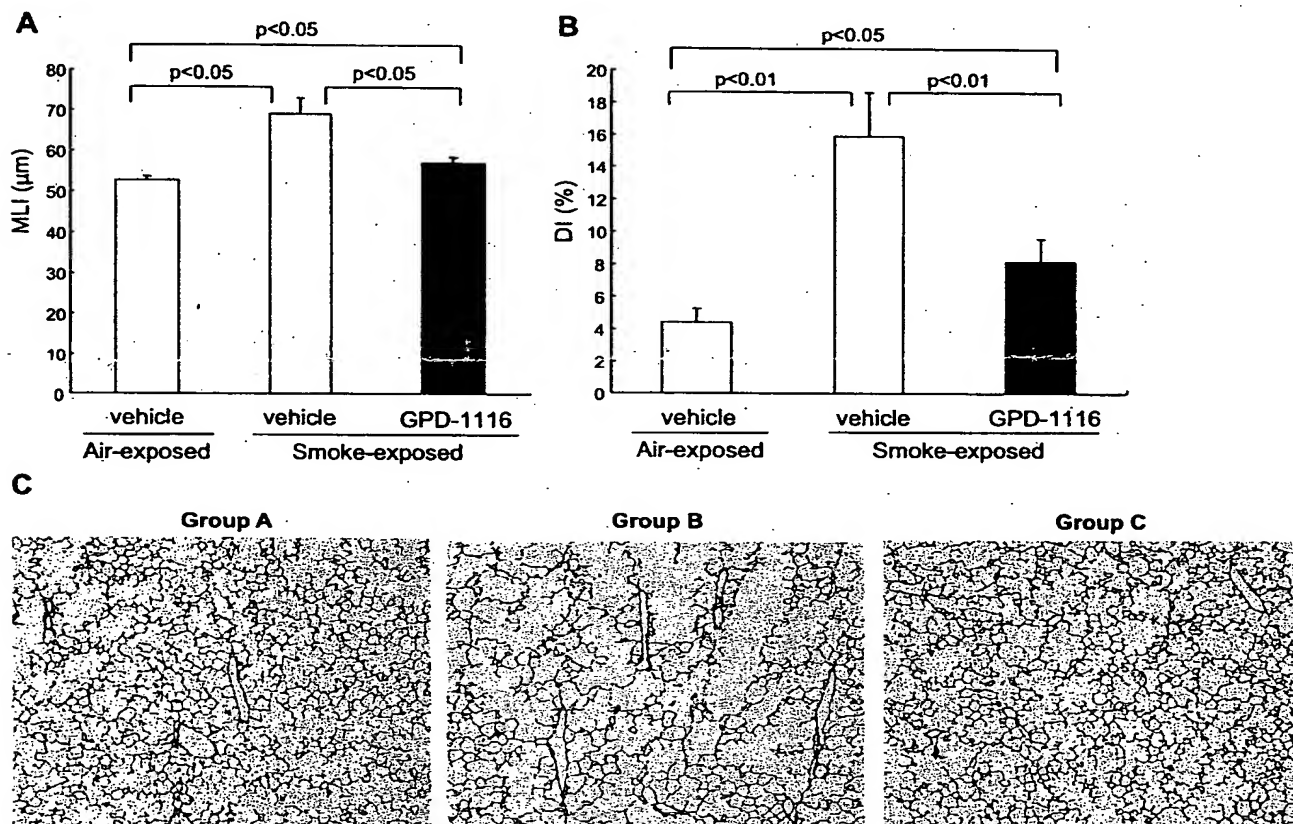


Fig. 3. A–C: morphometric and histological findings of the lung tissues of SAMP1. A: mean linear intercept (MLI). Values are presented as means \pm SE (μ m). Each group started at $n = 6$, but the measurement of MLI was actually done in the reduced number of mice (Group A, $n = 4$; Group B, $n = 5$; and Group C, $n = 5$) for technical reasons (failure to prepare the properly embedded histological specimen). The MLI was significantly greater in the lungs exposed to cigarette smoke (Group B) than those exposed to air (Group A) (Group A vs. B, 52.9 ± 0.8 vs. 68.4 ± 4.2 μ m; $P < 0.05$). MLI increased to 29% in cigarette smoke-exposed mice compared with that of air-exposed mice. On the other hand, the increase of MLI was significantly prevented (74% reduction) in the GPD-1116-treated mice (Group C, 57.0 ± 1.4 μ m; $P < 0.05$). B: destructive index (DI). Values are presented as means \pm SE (%). Each group started at $n = 6$, but the measurement of DI was actually done in the reduced number of mice (Group A, $n = 4$; Group B, $n = 5$; and Group C, $n = 5$) for technical reasons (failure to prepare the properly embedded histological specimen). Cigarette smoke exposure resulted in the increase of DI (Group A vs. B, $4.5\% \pm 1.3\%$ vs. $16.0\% \pm 0.4\%$; $P < 0.01$), but with the concurrent administration of GPD-1116, the destruction of the lungs was prevented from chronic cigarette smoke exposure (Group C, $8.2\% \pm 0.6\%$; $P < 0.01$). C: representative histological microphotographs are presented (hematoxylin and eosin stain; original magnification, $\times 20$). Compared with air-exposed mice (Group A), chronic cigarette smoke exposure for 8 wk generated marked air space enlargement (Group B), but with concurrent oral administration of GPD-1116, the mice lungs were prevented from developing emphysema (Group C).

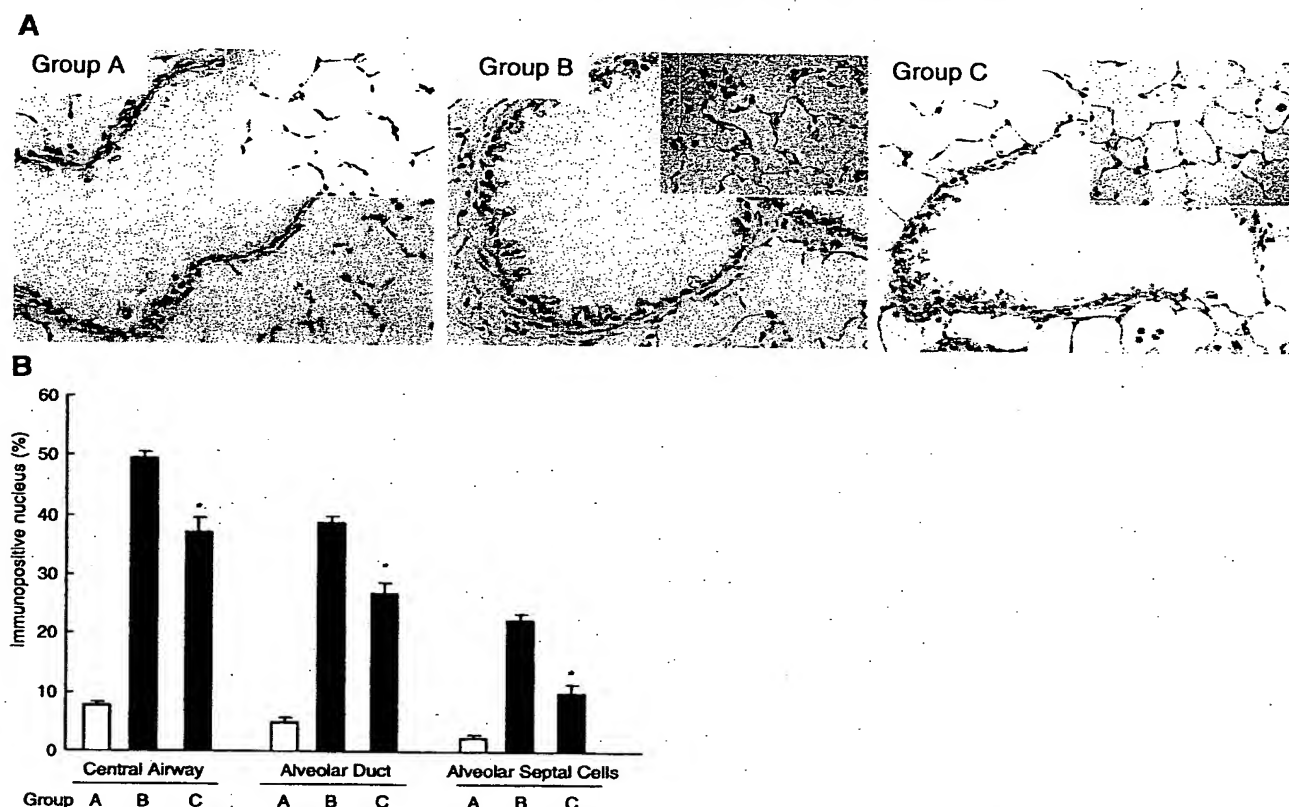


Fig. 4. **A** and **B**: immunohistochemical detection of apoptosis in the lungs after chronic cigarette smoke exposure. **A**: the representative histological microphotographs of immunohistochemistry for single-stranded DNA (ssDNA) are presented (original magnification, $\times 200$). *Insets* are presentation of the representative alveolar region at the same magnification. Note that positive immunostaining (nuclear brown staining) for DNA strand breaks was revealed in the airway epithelium and alveolar wall cells of the cigarette smoke-exposed lung (Group B) compared with the air-exposed lung (Group A) and that with concurrent administration of GPD-1116, marked decrease in positive immunostaining of lung cells was revealed in cigarette smoke-exposed lungs (Group C). **B**: immunoreactive nuclei for anti-ssDNA antibody were determined in 3 areas (bronchial cells in the central airway, bronchiolar cells adjacent to the alveolar duct, and alveolar septal cells in the parenchymal tissues) and expressed as the positive ratio (%) of total nuclei counted. Values are presented as means \pm SE ($n = 6$ for each group). * $P < 0.01$, data of Group C was significantly decreased compared with the cigarette smoke-exposed mice (Group B).

parenchymal area, GPD-1116 significantly reduced the percentage of apoptotic nuclei in all three areas (Group C) (central airway area, 10.1% reduction; alveolar duct area, 20.9%; and parenchymal area, 50.8%, respectively) compared with those of cigarette smoke-exposed SAMPl mice (Group B) but did not revert to the level of the air-exposed SAMPl (Group A, Fig. 4B).

Determination of the MMP-12 activity in the BALF and the supernatant of RAW264.7 cells. We next determined the MMP-12 activity in BALF, a major MMP produced by alveolar macrophages, since macrophages were predominant cells in BALF of SAMPl mice, and neutrophils were not detected even after 8-wk exposure to cigarette smoke. Casein zymography detected MMP-12 activity in the BALF that was increased after cigarette smoke exposure but decreased by concurrent oral administration of GPD-1116 (Fig. 5A). Quantification of MMP-12 activity by densitometry demonstrated that cigarette smoke exposure significantly increased MMP-12 activity (Group A vs. B, 4.1 ± 1.1 vs. 40.5 ± 16.2 area/ μ g protein, means \pm SE) but that GPD-1116 significantly decreased the MMP-12 activity in BALF (Group C, 5.3 ± 2.1 area/ μ g protein, Fig. 5B).

To investigate the direct effect of cigarette smoke on the production of MMP-12 by macrophages, we cultured RAW264.7 cells instead of alveolar macrophages from SAMPl mice since it was difficult to collect sufficient number of alveolar macrophages for this purpose. When RAW264.7 cells were cultured with 20% CSE for 72 h, MMP-12 activity in the culture supernatant significantly increased (control vs. 20% CSE, 259.3 ± 145.5 vs. $5,765.6 \pm 700.2$ area; $P < 0.01$), but GPD-1116 significantly inhibited CSE-induced increase of MMP-12 activity ($3,085.3 \pm 659.9$ area; $P < 0.05$; Fig. 6, A and B). Intracellular cAMP content did not change significantly among RAW264.7 cells cultured under the three different conditions described above when GPD-1116 was added at the start of cell culture and RAW264.7 cells were thereafter cultured for 72 h (data not shown). However, when GPD-1116 was added at 5 min before harvesting of the cells after 72 h culture under the three different conditions, we demonstrated that GPD-1116 significantly increased cAMP content in 20% CSE-stimulated RAW264.7 cells (9.77 ± 1.00 pg/mg protein, $P < 0.01$ compared with control or 20% CSE alone, 1.83 ± 0.47 pg/mg protein, Fig. 6C). The cAMP content in RAW264.7 cells tended to increase with CSE but was not statistically

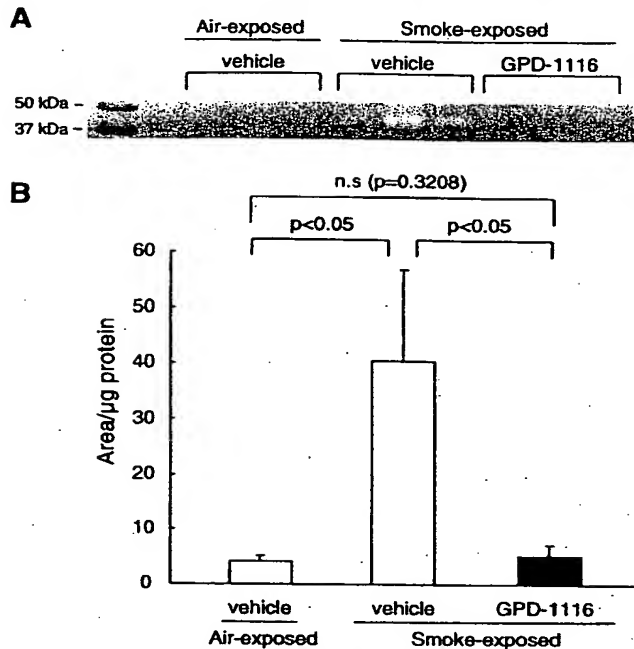


Fig. 5. *A* and *B*: matrix metalloproteinase-12 (MMP-12) activity in BALF determined with casein zymography. *A*: representative results of casein zymography are presented. Note that MMP-12 activity (45 kDa) increased in BALF (Group B) compared with Group A, and with concurrent administration of GPD-1116, the increase of MMP-12 activity in BALF was prevented (Group C). *B*: quantification of MMP-12 activity with densitometry. Values are presented as means \pm SE ($n = 6$ for each group). MMP-12 activities increased in BALF from cigarette smoke-exposed mice (Group B) compared with air-exposed mice (Group A), but with concurrent administration of GPD-1116 (Group C), decrease of MMP-12 activities in BALF to the same level of those from the air-exposed mice was revealed (Group A). n.s., not significant.

significant (control vs. 20% CSE, 0.23 ± 0.03 vs. 1.83 ± 0.47 pg/mg protein; $P = 0.0766$). These results indicate that CSE affects intracellular cAMP metabolism of RAW264.7 cells and can be modulated by GPD-1116.

Determination of VEGF in lung tissues and BALF. VEGF in the lung homogenate decreased after cigarette smoke exposure (Group B) as reported in our previous study (9) (Group A vs. B, 367.1 ± 4.5 vs. 228.6 ± 9.1 pg/mg protein, means \pm SE; $P < 0.01$), but GPD-1116 could not prevent the decrease of VEGF induced by smoke exposure (Group C, 226.7 ± 10.2 pg/mg protein; $P = 0.4449$ compared with Group B; Fig. 7A). As for BALF, VEGF concentration tended to decrease after cigarette smoke exposure (Group A vs. B, 164.2 ± 11.6 vs. 109.2 ± 32.8 pg/mg protein, means \pm SE; $P = 0.0876$), and GPD-1116 appeared to prevent the decrease of VEGF induced by cigarette smoke exposure (Group C, 220.5 ± 62.4 pg/mg protein; $P = 0.0765$), but no statistical significance was demonstrated between each SAMP1 group (Fig. 5B), probably due to the large variation that existed in Groups B and C.

DISCUSSION

We have shown in the present study that GPD-1116, a novel PDE4 inhibitor, markedly attenuated the development of cigarette smoke-induced emphysema in SAMP1 mice lungs when administered to mice during chronic smoke exposure for 8 wk.

GPD-1116 inhibited the increase of MMP-12 activity induced by chronic inhalation of cigarette smoke as well as MMP-12 production from RAW264.7 cells directly stimulated by CSE.

Before investigating the inhibitory effect of the PDE4 inhibitor in our chronic cigarette smoke exposure model, we

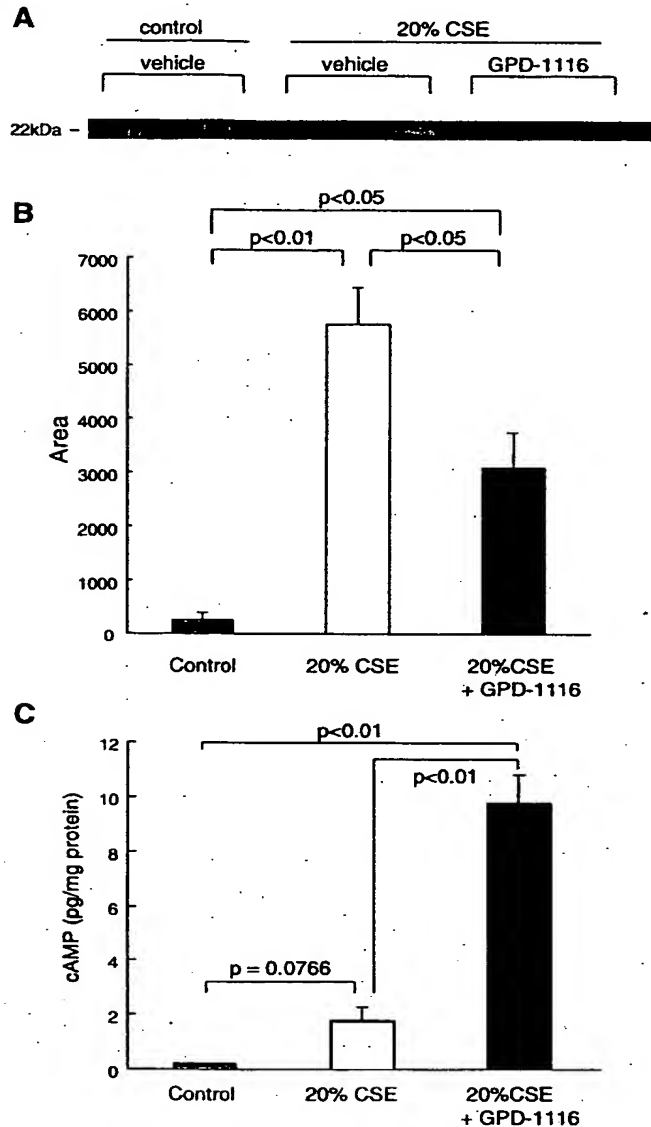


Fig. 6. *A–C*: MMP-12 activity in the culture supernatant of RAW264.7 cells (murine monocyte/macrophage cell line) determined with casein zymography. *A*: casein zymography revealed that, in contrast with BALF from SAMP1 mice, MMP-12 activity detected was mainly of those with the molecular size of 22 kDa, another active form of murine MMP-12. MMP-12 activity (22 kDa) increased in the cell culture supernatant when RAW264.7 cells were treated with 20% cigarette smoke extract (CSE), but GPD-1116 prevented the increase of MMP-12 activity in the supernatant when RAW264.7 cells were cotreated with 20% CSE and 1 μ M GPD-1116. *B*: quantification of MMP-12 activity with densitometry. Values are presented as means \pm SE ($n = 3$). *C*: determination of cAMP content in RAW264.7 cells. Values are presented as means \pm SE ($n = 3$). RAW264.7 cells were cultured for 72 h, and GPD-1116 at 1 μ M was added 5 min before the termination of cell culture.

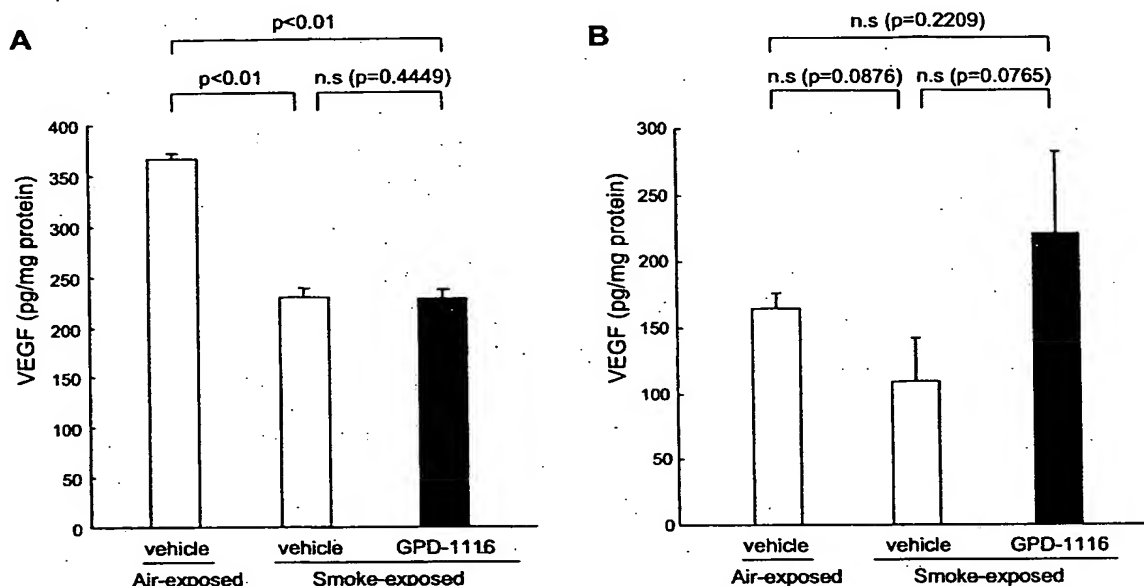


Fig. 7. *A* and *B*: VEGF concentration in lung tissues and BALF. *A*: lung tissues. Values are presented as means \pm SE ($n = 6$ for each group). VEGF content in the lung tissues decreased significantly after cigarette smoke exposure for 8 wk (air vs. smoke, 367.1 ± 4.5 vs. 228.6 ± 9.1 pg/mg protein; $P < 0.01$), but the concurrent oral administration of GPD-1116 failed to prevent the decrease of VEGF in the lung tissues (226.7 ± 10.2 pg/mg protein). *B*: BALF. Values are presented as means \pm SE ($n = 6$ for each group). VEGF tended to decrease in BALF from cigarette smoke-exposed mice (Group B, 109.2 ± 32.8 pg/mg protein) compared with that from air-exposed mice (Group A, 164.2 ± 11.6 pg/mg protein) and tended to be restored with concurrent administration of GPD-1116 (Group C, 220.5 ± 62.4 pg/mg protein). However, there was no statistical difference observed due to considerable variation of data in Group C.

evaluated the effect of GPD-1116 in an acute inflammatory model induced by LPS aerosol to determine a suitable dose of GPD-1116 for the chronic model. As shown in Fig. 2, GPD-1116 exerted a significant inhibitory effect on neutrophil influx into the lungs, however, its inhibitory effect on TNF- α production was somewhat weak, and thus 1 mg/kg GPD-1116 in SAMP1 mice was considered not to be sufficient to prevent chronic inflammatory events. Accordingly, we decided to treat mice with twice the amount of the dose used in the acute inflammation model, i.e., 2 mg/kg GPD-1116, in the experiment for chronic cigarette smoke exposure.

Cigarette smoke is a major risk factor for the development of COPD, directly and indirectly injures lung cells, and elicits abnormal inflammatory responses in the lungs. Inflammation involves the recruitment of inflammatory cells, such as neutrophils, macrophages, monocytes, and lymphocytes, and the activation of these cells to produce various inflammatory mediators and proteases in COPD patients. Several studies reported that intracellular cAMP level increased in alveolar macrophages of COPD patients (3, 4). Functional activities of alveolar macrophages are largely associated with intracellular cAMP level, and high levels of cAMPs coincide with down-regulation of functional activities (5). In this context, a drug exerting anti-inflammatory action to a variety of cells like a selective PDE4 inhibitor would be needed to treat COPD. For example, Cilomilast, one of the most potent and advanced PDE4 inhibitors, has shown some beneficial clinical effects in COPD patients, and larger studies are currently underway (6). Cilomilast is reported to inhibit collagen gel degradation induced by neutrophil elastase and TNF- α release *in vitro* (11). Roflumilast, another member of the potent and advanced PDE4 inhibitors, appears to be well-tolerated at doses that signifi-

cantly inhibit TNF- α release from peripheral blood monocytes (24). Recently, Roflumilast was demonstrated to prevent cigarette smoke-induced emphysema in a mouse model by ameliorating lung inflammations through augmentation of IL-10 in BALF (14).

In our mouse model for emphysema induced by chronic exposure to cigarette smoke, inflammation related to neutrophils and TNF- α appears not to play a critical role since neutrophil influx into BALF and increase of TNF- α in BALF were not detected as we have reported previously (9), although SAMP1 mice did demonstrate neutrophil influx and TNF- α production in BALF when exposed to aerosolized LPS as presented in Fig. 2. These findings may imply that SAMP1 mice demonstrate distinct response to inflammatory stimulus between acute and chronic phases. Since cell population in BALF was composed mainly of macrophages, and total cell number did not increase significantly even after chronic smoke exposure, we postulated that alveolar macrophages may be activated by direct and indirect effect of cigarette smoke exposure. Since mouse alveolar macrophages produce MMP-12, and cigarette smoke-induced emphysema was prevented in the MMP-12 knockout mice (8), we therefore evaluated MMP-12 activity in our mice model. We confirmed that chronic smoke exposure resulted in the increase of MMP-12 activity that could be inhibited by concomitant administration of GPD-1116. In addition, we found that MMP-12 overproduction from RAW264.7 cells directly stimulated by CSE was inhibited in the presence of GPD-1116. CSE indeed affected intracellular cAMP metabolism in RAW264.7 cells, and GPD-1116 markedly increased cAMP content in CSE-stimulated RAW264.7 cells. These results suggested that GPD-1116 pre-

vented the SAMP1 mice lungs from MMP-12-mediated destruction under chronic smoke exposure condition.

Cellular and molecular mechanisms involved in alveolar destruction are now rapidly evolving, and a new conceptual framework has recently been proposed (27, 28). In this concept, inflammation, apoptosis, and oxidative stress, those being evoked by inhalation of chronic cigarette smoke and the underlying aging process of the lungs, are intimately networked as mechanisms of alveolar destruction in emphysema. In our study, apoptosis of lung cells increased after chronic smoke exposure, and cigarette smoke-induced apoptosis was ameliorated with GPD-1116 administration but not completely prevented. Balance between lung cell apoptosis and cellular replenishment is an important system to maintain homeostasis of the lungs. VEGF is considered to play a central role in this issue since the blockade of signaling through VEGF receptor (10), genetic disruption of VEGF (20), and the generation of neutralizing antibody against VEGF or blocking antibody against the VEGF receptor (21) result in air space enlargement due to alveolar septal cell apoptosis. In our model, GPD-1116 could not restore cigarette smoke-induced decrease of the VEGF content in lung tissues, and no significant effect on the VEGF in BALF was observed. In our previous study (9), tomato juice containing a potent antioxidant lycopene fully restored the VEGF content in the lungs. Since oxidative stress and apoptosis are reported to interact through the VEGF signaling pathway (29), PDE4 inhibitor may be less potent in terms of modulating this interaction. Alternatively, the dose of GPD-1116 we administered to SAMP1 mice may not be sufficient enough to influence the potential interaction between smoke-induced inflammation and oxidative stress and apoptosis.

Emphysema is a major pathological finding of COPD together with chronic bronchitis and has to be considered with aging of the lungs since COPD is a disease of the elderly. The interaction between aging and cigarette smoke were illustrated by the several reports that, compared with never-smokers, telomere length in circulating lymphocytes significantly decrease with age in smokers with a dose-dependent manner of cumulative exposure to smoke (15) and that cigarette smoke-induced senescence of alveolar epithelial cells may impair re-epithelialization when certain injuries occur (25, 26). In this context, our animal model, SAMP1 strain, appears to have an advantage since the lungs have an intrinsic accelerated senescence and ensuing property of a senile lung (12, 18, 19, 22). According to the study by Kurozumi et al. (12), most of the morphometric parameters of the lung began to change from 2 mo of age and continue to progress up to 10 mo of age. In their study (12), SAMP1 mice at the age of 5 mo showed the same elastic recoil pressure of SAMR1, the control strain, at the age of 10 mo, suggesting that the lungs show accelerated senescence twice faster in SAMP1 mice than SAMR1 control mice (a mouse strain resistant to senescence) after maturation. We speculate that the mice at the age of 10 mo are analogous to human beings at age of young adults (30–40 yr old), based on the consideration where the lifespan of mice (~36 mo) and human beings (~100 yr) is assumed to be equal (personal communication with Dr. S. Goto, Tokyo Metropolitan Institute of Gerontology). We postulate that SAMP1 mice at 3–5 mo of age would be the appropriate age for use as the animal model in the experiment for smoke-induced emphysema since the

intrinsic mechanism(s) that cause(s) accelerated senescence would have been already initiated by the time of 2 mo of age, although SAMP1 mice at 3–5 mo of age appear to be younger (30–40 yr old in human beings) than COPD patients (usually 60–70 yr old in human beings). Moreover, as in human aging, SAMP1 mice lungs are postulated to have an intrinsic oxidative stress since the baseline level of glutathione content is increased in the lungs compared with that of SAMR1 control mice and could not be upregulated at all after smoke exposure (9). In addition, the system maintaining lung cell survival and structural integrity may also be impaired in SAMP1 mice since we demonstrated that the content of VEGF decreased after chronic cigarette smoke exposure for 8 wk in contrast with the same burden of smoke exposure resulting in an increase of the VEGF content in the lungs of the control mice (9). Although the precise genetic alteration(s) leading to accelerated senescence remain(s) unknown, our data suggest that SAMP1 mice is a useful animal model for smoke-induced emphysema to investigate not only its pathophysiology, but also to conduct an interventional experiment.

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GRANTS

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